

Identification of Phloem Involved in Assimilate Loading in Leaves by the Activity of the Galactinol Synthase Promoter¹

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The definition of "minor" veins in leaves is arbitrary and of uncertain biological significance. Generally, the term refers to the smallest vein classes in the leaf, believed to function in phloem loading. We found that a galactinol synthase promoter, cloned from melon (*Cucumis melo*), directs expression of the *gusA* gene to the smallest veins of mature *Arabidopsis* and cultivated tobacco (*Nicotiana tabacum*) leaves. This expression pattern is consistent with the role of galactinol synthase in sugar synthesis and phloem loading in cucurbits. The expression pattern in tobacco is especially noteworthy since galactinol is not synthesized in the leaves of this plant. Also, we unexpectedly found that expression in tobacco is limited to two of three companion cells in class-V veins, which are the most extensive in the leaf. Thus, the "minor" vein system is defined and regulated at the genetic level, and there is heterogeneity of response to this system by different companion cells of the same vein.

The phloem of mature leaves has two overlapping functions: loading photoassimilate from the mesophyll and exporting this material out of the lamina. Although loading is traditionally associated with the minor vein network, a clear distinction between major and minor veins has not been made. It is not known if such a distinction exists at the physiological, developmental, or regulatory levels or if the loading function simply diminishes in a gradual way in veins of larger order.

In anatomical terms, minor veins are defined as those without ribs of parenchyma tissue projecting beneath the lower leaf surface (Esau, 1965). Although convenient, this convention says little about the function of the veins, and different species have more or less distinct ribs. On a more functional but still arbitrary basis, minor veins are often considered to be those embedded in the mesophyll, without intervening parenchyma, which would presumably interfere with loading.

Another way to classify veins is to analyze their function(s) during development. In sink leaves, imported photoassimilate is unloaded from relatively large veins (class III in tobacco [*Nicotiana tabacum*]; Turgeon, 1987; Roberts et al., 1997) that delimit sizable islands of tissue. Within these islands, the network of smaller veins (classes IV–VI) is immature and non-functional. As the leaf grows and reaches positive carbon balance, import ceases, the smaller

veins mature, and phloem loading is initiated. It is not clear, as discussed above, that specific vein classes are devoted to the loading function.

An objective approach to identifying loading phloem is to localize proteins known to participate in this function. Since apoplastic phloem loading involves Suc-proton cotransport, the Suc symporter is one such candidate protein. Suc symporter genes are expressed in minor vein phloem (Riesmeier et al., 1993; Truernit and Sauer, 1995). However, Suc-proton symport is a common phenomenon in plant cells, and thus it is not surprising to find expression in other tissues, including the phloem of roots (Riesmeier et al., 1993) and the major veins and midribs of leaves (Truernit and Sauer, 1995).

Genes and proteins involved in the synthesis of raffinose and stachyose in leaves are more likely to mark the function of loading tissue. In plants that translocate substantial amounts of these sugars, such as the cucurbits, there is evidence that the synthesis of raffinose and stachyose is mechanistically linked to phloem loading (Turgeon, 1996). If true, expression of genes in this biosynthetic pathway should identify sieve element-companion cell complexes that participate in loading.

Galactinol synthase (GAS) is the first committed enzyme in the pathway leading to raffinose and stachyose synthesis (Keller and Pharr, 1996). In this study we cloned the GAS gene (*CmGAS1*) from melon (*Cucumis melo*) and studied the expression pattern that its promoter confers in *Arabidopsis* and cultivated tobacco. This pattern is consistent with the loading function: Gene expression is limited to the minor vein network. This is the case even in tobacco, which neither synthesizes galactinol in its leaves nor translocates either raffinose or stachyose. Thus, the

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CmGAS1 promoter appears to respond to a conserved regulatory system, with common elements between species, which delineates minor veins from larger vein orders and governs the loading function of leaf phloem. In addition the *CmGAS1* promoter is active in only two of the three companion cells in tobacco minor veins, signifying previously unrecognized heterogeneity among closely related sieve element-companion cell complexes in the same vein.

RESULTS

Cloning and Sequencing the GAS Promoter

A Southern blot from melon genomic DNA digested with *EcoRI* was probed with the 5' zucchini leaf *GAS* cDNA probe. Three bands were visible, suggesting that *GAS* is a small gene family in melon. However, there was only one strongly hybridizing band at 6 kb (not shown).

A melon sub-genomic library was created, and a clone containing a strongly hybridizing 6-kb *EcoRI* fragment was identified. The orientation of the clone was determined using probes derived from the 5' and 3' ends of the *CmGAS1* cDNA on blots of various restriction digests. This clone contained approximately 3 and 0.5 kb of non-hybridizing DNA at the 5' and 3' ends of the DNA respectively. The 3-kb upstream region was sequenced along with the start of the coding region. The gene was designated *CmGAS1* (GenBank accession no. AF249912).

Expression Patterns in Arabidopsis

To visualize expression conferred by the *GAS1* promoter, 3 kb of sequence upstream of the *CmGAS1* open reading frame was cloned upstream of the *gusA* gene in pBI101.2 to create pSG3K101. Arabidopsis plants transformed with pSG3K101 were selected on media containing kanamycin. DNA from each of nine positively transformed lines was digested with *BamHI*, and copy number was estimated by analysis of DNA blots probed with a 1-kb *BamHI/PstI* fragment from the *CpGAS1* promoter (not shown).

Mature leaves, siliques, flowers, and whole plants were incubated in β -D-glucuronide solution. Except where noted, results given are for low-copy-number lines; results from high-copy-number lines were the same except as discussed below. Wild-type controls were also stained following the same procedures; in no case was blue coloration observed in these tissues.

A typical shoot is shown in Figure 1A. Staining was very apparent in the veins of cotyledons and mature leaves. In younger leaves, minor vein staining began at the lamina tips and progressed basipetally as the leaves aged, in the same pattern as the sink-source transition of photoassimilate transport (Turgeon, 1989). Hydathodes also stained (Fig. 1A). In all cases,

β -glucuronidase (GUS) expression was absent or faint in the midrib and proximal ends of the secondary veins. Diffusion of reaction product was pronounced, especially in the cotyledons and first leaves, even though cyanide was included in the stain (Caissard et al., 1994).

To determine if the level of *gusA* expression was related to vein size, single leaves were cut into pieces and incubated in β -D-glucuronide solution for different times. At 3 h, staining was observed in the smallest veins and blind endings of the areoles (Fig. 1B) and became more evident in the larger veins at longer time periods (Fig. 1C). To facilitate penetration of substrate, similar experiments were conducted with leaf pieces cut 1 to 2 mm on a side. The same staining patterns were observed. Due to the diffusion of GUS reaction products, it was not possible to localize staining to individual cells in the phloem.

The time taken for stain to become visible in the veins, and ultimate staining intensity, were both proportional to transgene copy number. In high-copy-number plants, blue color was also detected in the mesophyll, although it was much less intense than in the veins and took more than 20 h to become apparent. Mesophyll staining was more apparent, and sometimes quite intense, in the cotyledons and in the first two true leaves than in the leaves subsequently produced. From these experiments, it cannot be established if this staining represents weak mesophyll expression in high-copy-number lines or diffusion from minor veins. It was also noted that damaged tissue demonstrated intense staining in the wounded area, presumably because GUS enzyme was released from the minor veins into the apoplast.

Veins in sepals and the vascular bundles of filaments were stained, veins in petals were faintly stained (Fig. 1D), but vascular bundles in the stem were unstained. Once sepals, petals, and filaments had abscised, the vascular bundle scars stained blue. However, the rest of the silique including seeds and remaining vasculature remained unstained (not shown). To be sure that stain was able to penetrate the silique we cut several longitudinally before staining; there was no difference in staining pattern. Light-blue color was apparent in the cortex and vascular bundles of hypocotyls, although it stopped at the boundary between the shoot and the root. Occasional roots were stained in light patches, most often in lateral roots or toward the apex of the primary root (Fig. 1E). Some of the patches were in the cortex, but more were in the vasculature. This staining was much lighter than in the leaves and took much longer to develop.

Expression Patterns in Tobacco

Growth on kanamycin was used to select for tobacco shoots transformed with pSG3K101. GUS staining was

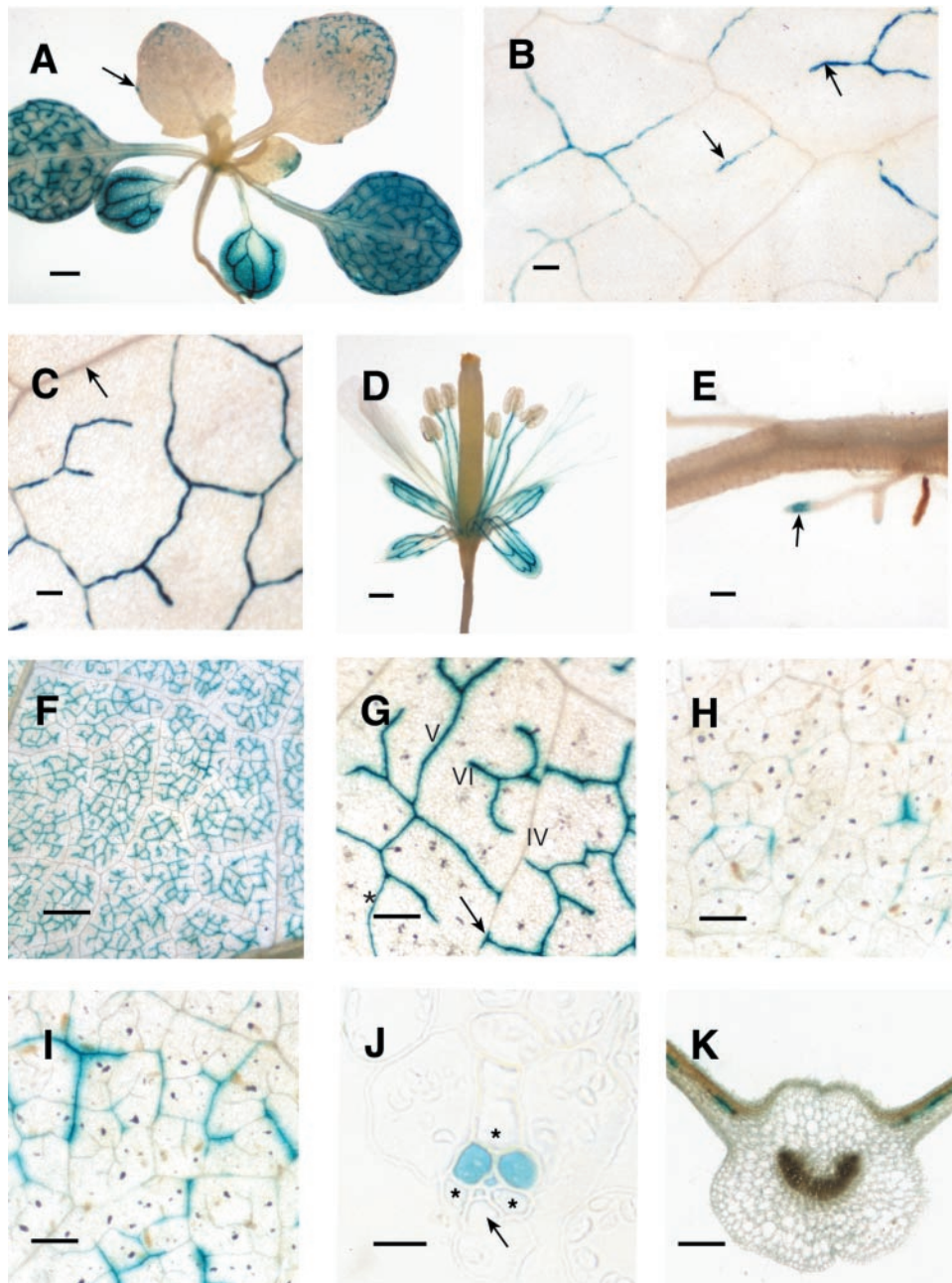


Figure 1. Staining patterns of *Arabidopsis* (A–E) and tobacco (F–K) transformed with the GAS promoter *CmGAS1-GUS* construct pSG3K101. A, Fourteen-day-old seedling. Staining is dense in the veins of the cotyledons and in most of the veins of the first-formed leaves. A progressive, basipetal (tip-to-base) staining pattern in minor veins is evident in the three developing leaves, the most mature of which is stained to approximately the midway point. Basipetal maturation is a characteristic of features associated with the sink-to-source transition in leaves. Hydathodes also stain (arrow). Bar = 1 mm. B, Mature leaf tissue stained for 3 h. Staining is most apparent in the blind endings (arrows) and other small veins. Bar = 200 μ m. C, After 24 h, all minor veins are stained. A secondary vein (arrow) is unstained. Bar = 200 μ m. D, In this flower, stain is visible in the filaments, in veins of the sepals, and less intensely in those of the petals. Bar = 0.5 mm. E, Faint blue staining near the tip of a lateral root. Bar = 0.5 mm. F, Leaf tissue near the tip of a growing leaf that has completed the sink-source transition. The vein in the lower right hand corner is the midrib, the one in the upper right is a secondary. Bar = 1.8 mm. G, Veins of a mature leaf. The vein classes are numbered. Note the partial staining of a class-IV vein at the junction of a class-V vein (arrow). There is no diffusion of stain from the vein marked with an asterisk. Bar = 400 μ m. H, Staining pattern of immature veins in the proximal region of a leaf undergoing the sink-source transition. Bar = 300 μ m. I, Tissue from the same leaf as H, but more distal and mature. Bar = 300 μ m. J, Localization of stain in a transverse section. Two companion cells, and the small sieve element between them, are stained. The third companion cell (arrow) and its sieve element are unstained. Phloem parenchyma cells are indicated by asterisks. Bar = 10 μ m. K, Transverse hand section through the midrib. Stained minor veins can be seen in the flanking lamina, but the vascular tissue of the midrib is unstained. Bar = 0.6 mm.

variable in the leaves of independent transformants, ranging from undetectable levels to easily discernable blue coloration in the minor veins (Fig. 1F). Expression patterns were studied in two independently derived, heavily staining transformants.

In mature leaves GUS activity was readily detected in the veins that define the areoles and in the blind endings of the areoles: class-V and -VI veins, respectively (Fig. 1F). Class-V veins are the most extensive in the leaf. Class-IV veins, which define larger islands of the vein network, were stained blue in some regions but not in others. Class-IV veins were often stained where class-V veins merged with them (Fig. 1G). Class-III veins, which define even larger segments of the vein network, class-I (midrib) veins, and class-II veins (branching from the midrib) did not stain. As in *Arabidopsis*, staining increased in intensity toward the finest veins. No staining was detected in mesophyll cells.

To determine if promoter activity is developmentally regulated, tissue was sampled from the tip to base of leaves undergoing the sink-source transition. In relatively immature tissue, staining was first evident in isolated patches of class-V veins, often at branch points (Fig. 1H). With increasing maturity, staining spread to more extensive regions of class-V veins (Fig. 1I), then to class VI veins, and to isolated regions of class-IV veins. Staining became progressively more intense in the more distal (mature) regions of the leaf.

To look for staining in other regions of the plant, hand sections were taken from different tissues of the progeny of transformants, from seedling to flowering stages, and stained with GUS substrate without cyanide. Staining was not apparent in veins of leaves larger than class IV or in the midrib (Fig. 1K). No stain was detected in the apical meristem or in axial meristems. However, in mature flowers, some but not all of the smaller veins of the sepals were blue (data not shown). No staining was present in any other tissues of mature flowers, including those of petals, stamens, carpels, or peduncles. Stem samples were free of stain except that in one sample of an older stem, irregular patches of stain were evident in the cortex and pith, especially the former. This staining was much lighter in intensity than that in the minor veins. No stain was detected in the vascular tissue of the root system, but stain was often detected in the apical meristems of roots.

The basipetal pattern of *gusA* induction in maturing leaves of *Arabidopsis* and tobacco is similar to the sink-source transition of photoassimilate transport (Turgeon, 1989). The timing of initial GUS staining with respect to the onset of phloem loading was therefore studied in tobacco with radiolabeling experiments (Fig. 2). First, the position of the import-termination boundary in developing leaves was visualized by autoradiography after exposing mature

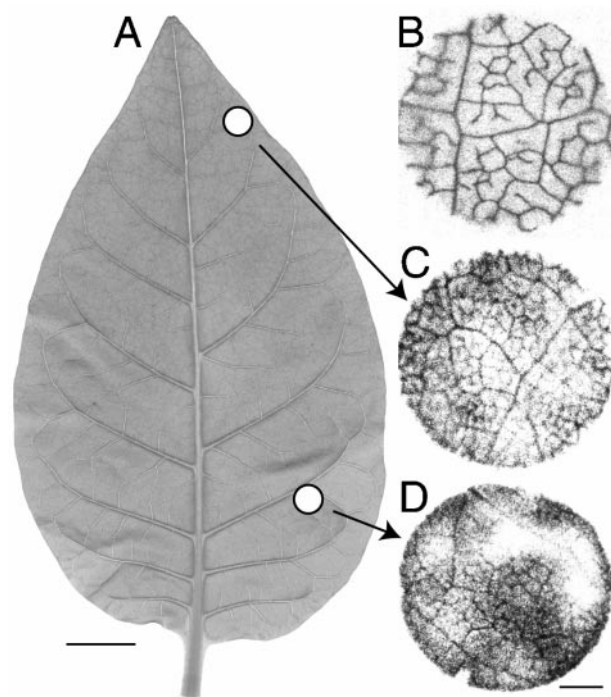


Figure 2. Phloem loading in leaf discs exposed to [^{14}C]Suc in plants transformed with the GAS promoter (GAS)-GUS construct pSG3K101. Due to less extensive uptake of label, discs in C and D were exposed to x-ray film for twice as long as the disc in B. A, Developing tobacco leaf the same size as the leaf used for the ^{14}C -labeling experiments. Leaves of this age have just ceased importing photoassimilate from mature leaves. In developing leaves there is a basipetal gradient in maturity, the distal end being more mature than the base. Bar = 1.5 cm. B, Autoradiograph of a disc from a fully expanded, mature leaf. The disc was exposed to a [^{14}C]Suc solution prior to freeze-drying. [^{14}C]Suc has accumulated in the veins. C, Autoradiograph of a disc excised near the tip of a developing leaf, as indicated by the arrow. [^{14}C]Suc has accumulated in the veins, although not to the same degree as in mature leaf tissue. GUS staining, as illustrated in Figure 1H, was visible from tissue adjacent to this disc but not in more proximal tissues. D, Autoradiograph of a disc excised near the base of a developing leaf, as indicated by the arrow. This is the most proximal tissue in which [^{14}C]Suc accumulated in the veins. No GUS staining was evident in adjacent tissue. Bar (in B–D) = 1 mm.

leaves to $^{14}\text{CO}_2$ (Turgeon, 1989; data not shown). Leaves of similar stages of development were then used for GUS-staining and phloem-loading studies. Discs of tissue were removed along the tip-to-base axis and were either stained for GUS or floated on [^{14}C]Suc solution. In the experiment shown in Figure 2, discs were removed from a leaf that had just stopped importing photoassimilate at its base. Weak, but nonetheless perceptible accumulation of radiolabel in veins was visible in the autoradiograph of a disc near the base of the leaf (Fig. 2D). Discs from more proximal tissue showed no evidence of loading, nor was there evidence of GUS staining in tissue from this region of the lamina. The first evidence of GUS staining, similar to that illustrated in Figure 1H, was visible near the leaf tip, where the tissue was

more mature. Phloem loading was more evident in tissue from this region (Fig. 2B) than from the base of the same leaf although still not as strong as in discs taken from fully expanded, mature leaves (Fig. 2A; corresponding GUS staining pattern in Fig. 1G).

GUS Expression in Different Companion Cells of Tobacco Minor Veins

Even when cyanide was included in the GUS solution, some diffusion of the stain was usually evident (Fig. 1, G–I). However, on close inspection, stain was better localized in some veins than in others. This can be seen in Figure 1G; there is no evidence of diffusion from the vein marked with an asterisk. We took advantage of this precise localization and the regular and relatively simple structure of tobacco minor veins to study expression in mature leaf tissue at the cell level. Stained tissue was fixed in glutaraldehyde, cleared, and examined with a stereo microscope. Veins with precise stain localization were excised and embedded for microscopy.

In tobacco, the minor vein phloem of class-V veins consists of three parenchyma cells that alternate in a ring with three companion cells, surrounding two sieve elements (Ding et al., 1988, 1995, 1998). In veins such as the one indicated in Figure 1G, GUS stain was restricted to the two adaxial companion cells (Fig. 1J). No staining was detected in the abaxial companion cell or in the parenchyma cells in any of the veins examined. This staining pattern was consistent in the progeny of two independent transformants, whether the lower epidermis had, or had not been removed before staining.

Absence of Galactinol Synthesis in Tobacco

To determine if mature tobacco leaves synthesize galactinol, attached leaves were exposed to $^{14}\text{CO}_2$ for 5 min and then to room air for a further 25-min chase before extraction. No galactinol spot was present on autoradiographs of thin-layer chromatography plates. In one quantitative analysis, the spot on the thin-layer chromatography plate corresponding to Suc contained 1.47×10^5 Bq, whereas no activity above background (0.8 Bq) was detected in the galactinol spot.

DISCUSSION

In anatomical terms, minor veins do not have rib tissue that protrudes beneath the surface of the lamina (Esau, 1965). From a physiological and developmental perspective, minor veins are those that are immature in sink leaves and do not participate in phloem unloading (Turgeon, 1987; Roberts et al., 1997) but mature during the sink-source transition (Turgeon and Webb, 1976) and are believed to be the predominant site of photoassimilate loading into the

translocation stream. The anatomical, developmental, and functional roles of leaf venation have been well studied in tobacco. The veins of tobacco leaves have been subdivided into classes, based on cell numbers rather than branching pattern, which can be misleading (Ding et al., 1988).

The different vein classes in tobacco appear to have specific roles, to a certain degree, in photoassimilate unloading and loading. Class-III veins define large sectors of the lamina and are responsible for most photoassimilate unloading in sink leaves (Turgeon, 1987; Roberts et al., 1997). Class-III veins probably have at most a limited role in loading once the leaf is mature since they comprise a relatively small proportion of total vein length and the phloem is separated from mesophyll cells by several layers of parenchyma (Ding et al., 1988). Class-IV veins define smaller, but still relatively large islands of lamina and have one layer of intervening parenchyma in most cases (Ding et al., 1988); thus they lack the required cumulative length to have more than a limited role in phloem loading. It appears that most phloem loading occurs in class-V veins, based on their great cumulative length and the fact that they outline almost all the areoles. Further, class-V veins lack the layer of parenchyma found in larger vein classes, which could impede transport of carbohydrates from mesophyll to the sieve tubes. This is not to say that a small amount of phloem loading could not occur in larger vein classes; apoplastic phloem loading apparently involves the recruitment of a general mechanism of Suc retrieval present in most if not all cells. Therefore, there is a potential for flux of Suc into the phloem all along the transport pathway.

Arabidopsis veins differ from those of tobacco in that there are fewer branching orders and a larger proportion are apparently devoted to phloem loading. All but the largest veins are embedded directly in the mesophyll without intervening layers of parenchyma (Haritatos et al., 2000). Furthermore, only the first and second orders of veins are involved in phloem unloading (Imlau et al., 1998).

It seemed reasonable that the *CmGAS1* promoter would confer gene expression specifically in the subset of minor veins where phloem loading occurs. GAS catalyzes the first committed step leading to the production of raffinose and stachyose for export. The synthesis of these sugars is apparently an integral part of the phloem-loading mechanism in cucurbits and other plants with intermediary cells (Turgeon, 1996). Intermediary cells are specialized companion cells of minor veins that are found only in plants that export raffinose-family oligosaccharides. In leaves, synthesis of raffinose-family oligosaccharides takes place in intermediary cells but not in companion cells of major veins or in the “ordinary” companion cells of minor veins (Holthaus and Schmitz, 1991; Beebe and Turgeon, 1992). To determine the tissue and cell specificity of the *CmGAS1* promoter, we cloned 3 kb

of sequence upstream of the GAS open reading frame and analyzed the expression pattern this element confers on the *gusA* reporter gene in Arabidopsis and tobacco.

The observed expression pattern correlates well with the size of veins thought to be actively involved in phloem loading. As discussed above, there may be some phloem loading, or at least retrieval of leaked Suc, in larger veins. Indeed, the promoter of the Arabidopsis SUC2 Suc-H⁺ symporter directs expression to source leaf phloem and also to the phloem of larger veins and other tissues (Truernit and Sauer, 1995). The unique feature of the *CmGAS1* promoter is that the conferred expression pattern reveals the subset of minor veins that appear, on the basis of overall length and structural characteristics, to be most actively devoted to the loading function.

How well does the expression pattern of the *CmGAS1-GUS* construct correlate with phloem loading activity? Unfortunately, it is not possible to answer this question with certainty: Transport along the phloem is rapid and when loading studies are conducted with radiolabeled sugars or ¹⁴CO₂, the label quickly disperses throughout veins of all sizes. It is known from radiolabeling studies that there is a substantial lag between the termination of import and the beginning of export in leaf tissue undergoing the sink-source transition (Turgeon and Webb, 1975). Since *CmGAS1* is involved in export, it was expected that *CmGAS1-GUS* expression would not correlate with cessation of import. However, there also appears to be a lag between the onset of phloem loading in tobacco leaves, as revealed by [¹⁴C]Suc accumulation, and the initial expression of the *CmGAS1-GUS* construct. This lag may be due, in part, to differences in sensitivity between the autoradiographic technique and GUS staining. Alternatively, since the *CmGAS1* promoter is heterologous in tobacco, a greater accumulation of transacting factors may be required for activation. It should be possible to distinguish between the contributions of these factors to the lag period once melon plants transformed with *CmGAS1-GUS* become available.

Within class-V tobacco minor veins, the promoter is active in only two of six cells that customarily surround the two sieve elements. The two cells in these positions have been identified as companion cells since they do not plasmolyze when the tissue is incubated in 1 M sorbitol (Ding et al., 1995, 1998). However the lower (most abaxial) cell in the vein is also considered to be a companion cell by the same criterion, and it does not stain in the minor veins of two lines of tobacco independently transformed with *CmGAS1-GUS*. Therefore, the promoter is either differentially expressed in the various companion cells of the same vein, or the most abaxial of these cells is not a true companion cell. The latter interpretation seems unlikely since the lower of the two sieve elements usually appears to be in close juxtaposition

with the cell in question, thus implying an ontogenetic relationship. It is worth noting that GAS is immunolocalized to intermediary cells of cucurbits, but not to "ordinary" companion cells in the same minor veins (Beebe and Turgeon, 1992), a finding that is also consistent with highly regulated, differential expression of the gene for this enzyme in closely associated companion cells.

Since the *CmGAS1* promoter from melon confers a similar expression pattern in Arabidopsis and tobacco, it appears to respond to conserved transacting signals. This is true even though the amount of galactinol produced by these three species differs considerably. Melon transports high levels of raffinose and stachyose and produces almost as much galactinol as raffinose (Haritatos et al., 1996). Arabidopsis translocates only a small amount of raffinose and synthesizes less galactinol than melon (Haritatos et al., 2000). We could not detect synthesis of any galactinol in tobacco. This suggests that the transacting factor(s) that activate the *CmGAS1* promoter in tobacco regulate the expression of other genes involved in companion cell function and photoassimilate export.

Significantly, these findings indicate that the somewhat arbitrary definition of a "minor vein" network within the leaf is supported at the molecular genetic level. That is, different vein classes and different companion cells within the same vein apparently have unique compliments of transacting factors and consequently have distinct cascades of gene expression. Since the phloem network is central to the growth and development of plants, this genetic heterogeneity deserves further study.

MATERIALS AND METHODS

Plant Material

Melon (*Cucumis melo* cv Hale's Best Jumbo; Vaughan's Seed Company, Downers Grove, IL), Arabidopsis var Columbia, and cultivated tobacco (*Nicotiana tabacum* cv Petit Havana SR1) were used in this study. For melon DNA extraction, seed coats were removed, and seeds were germinated on damp filter paper in the dark for 7 to 9 d at 24°C. Arabidopsis plants were grown in artificial soil in 10-cm plastic pots covered with nylon window screen in a controlled-environment chamber with a 16-h light/8-h dark cycle at 21°C. Plants were fertilized twice a week with Peters 20:20:20 fertilizer (Scotts-Sierra Horticultural Products, Marysville, OH). Tobacco plants were grown under similar conditions but at higher temperatures (25°C).

DNA Extraction and Southern Blotting

Genomic DNA was extracted from dark-grown melon seedlings by crude pelleting of nuclei (Bingham et al., 1981) followed by a cetyltrimethylammonium bromide extraction procedure (Bernatzky and Tanksley, 1986). DNA was further purified by cesium chloride equilibrium density

gradient centrifugation (Ausubel et al., 1995). Cesium chloride was removed from the DNA by dialysis against Tris (tris[hydroxymethyl]aminomethane)-EDTA buffer, pH 8.0 (Sambrook et al., 1989). For *Arabidopsis*, DNA was extracted from young leaves using a miniprep procedure.

Melon genomic DNA was digested with *EcoRI* restriction endonuclease and resolved by agarose gel electrophoresis. Southern blotting and hybridization were conducted using standard procedures (Sambrook et al., 1989). *GAS*-specific probes were constructed from a zucchini leaf *GAS* cDNA (*CpGAS1*), kindly provided by DuPont (Wilmington, DE; Kerr et al., 1992). The *CpGAS1* cDNA was originally isolated from a zucchini leaf cDNA expression library and shown to have *GAS* catalytic activity (Kerr et al., 1992). A 3' *GAS* probe was made from an 0.8-kb *HindIII* fragment that included 697 bases of coding region and 90 bases downstream of the translational stop site. A 5' *GAS* probe was made from a 0.3-kb *XbaI/HindIII* fragment that included 37 bases upstream of the translational start site and 300 bases of coding region. Probes were labeled with [α - 32 P]dCTP (DuPont-New England Nuclear, Boston) using a random-primed DNA labeling kit (Boehringer Mannheim, Indianapolis), and unincorporated nucleotides were removed by passage over a microspin column filled with Sephadex G50 (Sigma, St. Louis). Membranes were exposed to film (Jersey Lab Supply autoradiography film, Jersey Lab Supply, Livingston, NJ), and autoradiographs were developed using standard procedures.

Subgenomic Library

From a melon genomic DNA blot, a potential *GAS* gene was identified on a 6-kb *EcoRI* fragment. Fifty micrograms of DNA was digested to completion with *EcoRI* and the resulting fragments separated on a 0.68% (w/v) Seaplaque GTG low-Tm agarose gel (FMC BioProducts, Rockland, ME). Size fractionated fragments between 5 and 7 kb were isolated by electro-elution (Sambrook et al., 1989) and ligated to *EcoRI*-cut and CIAP-treated λ gt11 arms (Stratagene, La Jolla, CA) in a molar ratio of approximately 1:1. The ligation reaction was packaged in vitro using Gigapack III Gold packaging extract (Stratagene) according to the manufacturer's instructions.

Phages were incubated with *Escherichia coli* strain Y1088 and plated onto 150-mm NZY plates according to the manual provided (Stratagene). Lifts were made onto Magna Lift T nylon membranes (Micron Separations, Westboro, MA). The 5' *GAS* probe described above was used to screen the library, and membranes were washed at high stringency (0.2 \times SSC, and 0.5% SDS, 50°C). Positive plaques from the first screen were purified by two additional rounds of screening using the same probe. Melon DNA from positive clones, isolated as *EcoRI* fragments, was subcloned into pBluescript II KS⁺ (Stratagene) digested with *EcoRI*. Positive clones contained the same 6-kb *EcoRI* segment of melon DNA. This clone, designated pSG8E, was sequenced at the Cornell DNA sequencing facility using a Perkin Elmer/Applied Biosystems Division 377 Automated DNA

Sequencer, Dye Terminator chemistry, and AmpliTaq-FS DNA polymerase (Perkin-Elmer Applied Biosystems Division, Foster City, CA).

Vector Construction

A putative translational start site was identified in the *CmGAS1* sequence by comparison to the *CpGAS1* cDNA sequence (Kerr et al., 1992). The subgenomic *CmGAS1* clone pSG8E was cut with *XbaI* (3,082 bases upstream of the putative translational start site) and *PvuII* (13 bases downstream of the putative translational start site). This fragment was ligated into the *XbaI* and *SmaI* sites of pBI101.2 (a binary vector with a promoterless *gusA* gene; CLONTECH Laboratories, Palo Alto, CA) to create pSG3K101. Since the *SmaI* site of pBI101.2 is 17 bases upstream of the *gusA* translational start site, the *gusA* sequence is in-frame with the *CmGAS1* start codon.

Plant Transformation

Plasmid pSG3K101 was introduced into competent *Agrobacterium tumefaciens* strain GV3101 by a freeze-thaw procedure (Hooykaas, 1988). Stable transformants of *Arabidopsis* were obtained by infiltration of the plants with a suspension of *A. tumefaciens* as described by van Hoof and Green (1996), except that no vacuum was used. Instead, rosettes and inflorescences were immersed in the *A. tumefaciens* suspension for 15 min. Plants were self-fertilized, and the resultant seed was plated on Murashige and Skoog medium containing 50 μ g/mL kanamycin, with either 500 μ g/mL vancomycin or 200 μ g/mL cefotaxime to inhibit growth of bacteria. Transformants were transferred to artificial soil and allowed to self-pollinate, and seed was collected and planted on artificial soil for assays of reporter gene activity.

Tobacco cv Petite Havana SR1 was transformed by cocultivation of leaf discs with *A. tumefaciens* harboring pSG3K101 essentially as described by Horsch et al. (1985). Except where noted, primary transformants were used for reporter gene assays.

Histochemical Localization of GUS Expression

GUS staining was performed using the substrate 5-bromo-4-chloro-3-indolyl β -D-glucuronide according to the method described by Jefferson (1987), but with 3 mM potassium ferri- and ferrocyanide added to limit diffusion of GUS reaction products (Caissard et al., 1994). Samples were incubated in β -D-glucuronide solution at 37°C for 20 h unless otherwise specified. After incubation, samples were cleared in ethanol. For resolution of specific cells, tissue was first stained and then fixed in glutaraldehyde by conventional techniques. The fixed tissue was dehydrated in ethanol and veins with good stain localization were identified, embedded in LR White resin, sectioned at 2- μ m

thickness, and observed under phase contrast without further staining.

Radiolabeling

For [^{14}C]Suc uptake studies, the adaxial surface of a leaf was abraded with carborundum, and leaf discs were removed with a cork borer under the surface of MES buffer (20 mM MES [2(*N*-morpholino)ethane-sulfonic acid] plus 2 mM CaCl_2 , pH 5.5, with NaOH). The discs were transferred, abraded side down, to the surface of fresh buffer containing [^{14}C]Suc (1 mM; 30 kBq mL^{-1}). Discs were incubated at room temperature on a shaker for 20 min, washed in three 10-min changes of fresh buffer at room temperature, and frozen in powdered dry ice. Frozen tissue was lyophilized in a -30°C chamber, pressed thin between steel plates in a large vice, and pressed against x-ray film (Hyperfilm- β max, Amersham, Buckinghamshire, UK). In photoassimilate-labeling studies, an attached leaf was enclosed in a Plexiglas cuvette and exposed to $^{14}\text{CO}_2$ generated in the barrel of a syringe from $\text{Na}_2^{14}\text{CO}_3$ (6.6×10^5 MBq mmol^{-1}). Five minutes later the cuvette was removed, and the leaf, still attached to the plant, was exposed to a 25-min chase in room air before being frozen in liquid nitrogen. Radiolabeled compounds were extracted, passed through ion-exchange resin (Turgeon et al., 1993), and analyzed by thin-layer chromatography as previously described (Turgeon and Gowan, 1992).

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